APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1991, p. 893–900
0099-2240/91/040893-08$02.00/0

Vol. 57, No. 4

Genetic Improvement of *Escherichia coli* for Ethanol Production: Chromosomal Integration of *Zymomonas mobilis* Genes Encoding Pyruvate Decarboxylase and Alcohol Dehydrogenase II†

KAZUYOSHI OHTA, D. S. BEALL, J. P. MEJIA, K. T. SHANMUGAM, AND L. O. INGRAM*
Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611

Received 27 August 1990/Accepted 3 December 1990

Zymomonas mobilis **genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) were integrated into the *Escherichia coli* chromosome within or near the pyruvate formate-lyase gene (*pfl*). Integration improved the stability of the *Z. mobilis* genes in *E. coli*, but further selection was required to increase expression. Spontaneous mutants were selected for resistance to high levels of chloramphenicol that also expressed high levels of the *Z. mobilis* genes. Analogous mutants were selected for increased expression of alcohol dehydrogenase on aldehyde indicator plates. These mutants were functionally equivalent to the previous plasmid-based strains for the fermentation of xylose and glucose to ethanol. Ethanol concentrations of 54.4 and 41.6 g/liter were obtained from 10% glucose and 8% xylose, respectively. The efficiency of conversion exceeded theoretical limits (0.51 g of ethanol/g of sugar) on the basis of added sugars because of the additional production of ethanol from the catabolism of complex nutrients. Further mutations were introduced to inactivate succinate production (*frd*) and to block homologous recombination (*recA*).**

Previous studies have demonstrated that recombinant *Escherichia coli* expressing plasmid-borne *Zymomonas mobilis* genes for pyruvate decarboxylase (PDC; *pdc*) and alcohol dehydrogenase II (ADHII; *adhB*) can efficiently convert both hexose and pentose sugars to ethanol (1, 13, 14, 22, 23). Ethanologenic *E. coli* strains require simpler fermentation conditions, produce higher concentrations of ethanol, and are more efficient than pentose-fermenting yeasts for ethanol production from xylose and arabinose (2, 16, 30).

Subsequent studies have examined the effects of environmental conditions on batch fermentations with these organisms (1, 23). Conversion efficiencies were very high, although appreciable amounts of organic acids were made as coproducts with ethanol. In these experiments, pH control was achieved primarily through the inclusion of 0.1 to 0.2 M phosphate buffer. Optimal conditions for fermentation were found to be between pH 6.0 and 7.0, at 30°C, and with xylose concentrations below 10% or glucose concentrations below 15%.

Extremely stable recombinant organisms and continuous fermentation systems may prove essential for the development of economically viable processes to produce high volumes of low-value chemicals such as ethanol. Although recombinant strains containing multicopy plasmids are often quite suitable for commercial application in batch processes for high-value products, these plasmid-based recombinants are typically less stable than strains in which the foreign genes have been integrated into the host chromosome. However, single copies of integrated genes may not produce the high levels of recombinant products achieved in recombinants containing multicopy plasmids.

In this study, we have integrated an artificial operon containing the fermentative genes (*pdc* and *adhB*) from *Z. mobilis* into the *pfl* region of the *E. coli* chromosome, conferring the ability to efficiently produce ethanol in the absence of plasmid-borne *Z. mobilis* genes.

High levels of PDC and ADHII are required for fermentation in *Z. mobilis* (15), and similarly high levels are essential for ethanol production by recombinant *E. coli* (13). The *pfl* gene encoding pyruvate formate-lyase (PFL) was selected as the integration site for three reasons. PFL represents a competing branch point (pyruvate to formate plus acetyl coenzyme A [CoA]) for the diversion of pyruvate away from homo-ethanol production in ethanologenic *E. coli* (7, 18). This gene is expressed at very high levels in *E. coli* during anaerobic growth from at least six promoters (28, 29). The *pfl* gene is well characterized, having been cloned (6) and sequenced (25).

Additional genetic modifications which eliminate succinate production (*frd*) and inactivate the *recA* gene are also described. Mutants defective in *recA* may be useful as hosts for the manufacture of recombinant proteins as coproducts during ethanol fermentation.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* strains and plasmids used in this study are listed in Table 1. Cultures were grown in Luria broth (19) supplemented with the indicated levels of glucose or xylose. Luria agar containing 2% glucose and appropriate antibiotics was used for the selection of recombinants. Antibiotic concentrations were as follows: ampicillin, 50 μg/ml; chloramphenicol (Cm), 20, 40, or 600 μg/ml; and tetracycline, 12.5 μg/ml. Expression of *Z. mobilis* ADHII in *E. coli* recombinants was screened with aldehyde indicator plates (9).

Genetic procedures and recombinant techniques. Standard procedures were used for plasmid preparations, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis (26). *E. coli* TC4 was used as the host for all plasmid constructions.

Inactivation of *pfl* by chromosomal integration of *Z. mobilis pdc* and *adhB* genes. *Z. mobilis pdc* and *adhB* were integrated into *E. coli* ATCC 11303 by using a derivative of the integration vector pMAK705 (11). The pSC101 replicon in

* Corresponding author.
† Florida Agricultural Experiment Station publication R00955.

TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli K-12		
TC4	*recA lacZY*	13
JC10240	Hfr PO45 *srl*::Tn*10* *recA*	L. Czonka
KL282	Hfr PO2A *ser*S *ser*C	B. Bachmann[a]
DW12	Δ(*frdABCD*)102 *zid*::Tn*10*	R. P. Gunsalus (3)
SE1706	KL282 Δ(*frdABCD*) *zid*::Tn*10*	This paper
E. coli B	Prototroph	ATCC 11303
KO1	*pfl*::(*pdc*$^+$ *adhB*$^+$)[b]	This paper
KO2	*pfl*::(*pdc*$^+$ *adhB*$^+$)	This paper
KO3	*pfl*$^+$ *pfl*::(*pdc*$^+$ *adhB*$^+$ Cm^r)	This paper[c]
KO4	KO3 selected for high Cm^r (600 μg/ml); also hyperexpressive for *pdc* and *adh*	This paper
KO5	Independently isolated, analogous to KO4	This paper
KO10	KO4 *recA*	This paper
KO11	KO4 *frd*	This paper
KO12	KO11 *recA*	This paper
KO20	Mutant of KO2 selected for high-level expression of *adhB*	This paper
Plasmid		
pUC19	*bla lacI'Z'*	BRL[d]
pHB4	*pfl act'* Ap^r	G. Sawers (28)
pBR325	Ap^r Cm^r Tc^r	24
pMAK705	Cm^r *lacZ' rep(ts)*	S. R. Kushner (11)
pLOI295	Ap^r *pdc adhB*	15
pLOI297	Tc^r *pdc adhB*	1

[a] CGSC 4297, Coli Genetic Stock Center, Yale University, New Haven, Conn.
[b] The *adhB* and *pdc* genes were isolated from *Z. mobilis*.
[c] A second functional *pfl* gene was also generated as a result of a single crossover event during homologous recombination.
[d] BRL, Bethesda Research Laboratories, Bethesda, Md.

this vector is temperature sensitive and does not function at 44°C (11). By using this vector, a plasmid (pLOI543) was constructed to include the *Z. mobilis* genes for ethanol production and an *E. coli* DNA fragment with the *pfl* coding region to guide homologous recombination (Fig. 1A). For this construction, pHB4 carrying an incomplete *E. coli* gene for pyruvate formate-lyase (*pfl*) was partially digested with *Bam*HI. Protruding ends were filled in by using the Klenow fragment of DNA polymerase I, and a *Sal*I linker (dCGTC GACG) was inserted to inactivate the *Bam*HI site and provide a second *Sal*I site. The two *Sal*I sites in pLOI153 allowed the excision of the *pfl'* portion. A 3.2-kbp DNA fragment containing promoterless *Z. mobilis pdc* and *adhB* genes was isolated from pLOI295 following digestion with *Eco*RI and *Sal*I. After treatment with the Klenow fragment of DNA polymerase, this fragment was ligated into the Klenow-treated *Bam*HI site within the *pfl'* gene of pLOI513. The construct, pLOI542, was selected in which *pfl'* and the *Z. mobilis* genes were oriented in the same direction with respect to transcription. A 7.2-kbp *Sal*I fragment from pLOI542 containing the *Z. mobilis* genes flanked by parts of

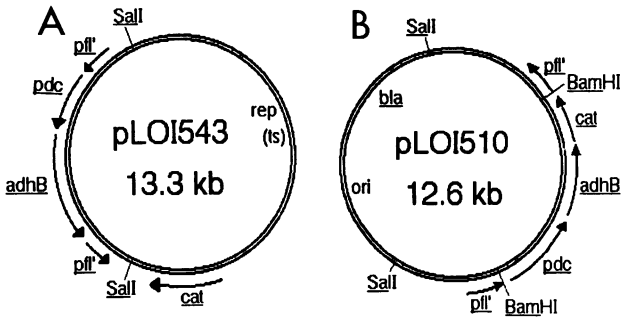

FIG. 1. Vectors constructed to allow chromosomal integration of the *Z. mobilis pdc* and *adhB* genes into the *pfl* gene of *E. coli*. (A) pLOI543, containing a temperature-conditional pSC101 replicon. (B) pLOI510, containing an excisable *Sal*I fragment which includes *cat* and *Z. mobilis pdc* and *adhB* between flanking regions of an *E. coli pfl* gene.

pfl was ligated into the *Sal*I polylinker site of the integration vector pMAK705 to produce pLOI543. This plasmid was used to transform strain ATCC 11303 with selection for resistance to Cm (20 μg/ml) at 30°C.

Conditions for the selection of two homologous recombination events were based upon the previously described procedure of Hamilton et al. (11). Single colonies of transformants were inoculated into 100-ml L-broth cultures containing 20 μg of Cm per ml and 5% glucose. These were grown for 24 h at 44°C to select for chromosomal integration. This culture was diluted 1:20,000 into a second 100 ml of L broth lacking antibiotic and incubated at 30°C for 12 h to allow excision. Two more cycles of growth at 30°C were carried out without antibiotic to enrich for the more rapidly growing cells which have lost the pSC101 replicon (11). The resulting suspension was diluted again into L broth and incubated for 12 h at 44°C to eliminate free plasmids. Appropriate dilutions were spread on Luria agar plates containing 2% glucose. Single colonies from plates incubated at 30°C were screened for expression of ADHII activity by using aldehyde indicator plates (9) and for sensitivity to 20 μg of Cm per ml to identify clones in which the vector (without *Z. mobilis* genes) had been deleted by a second recombinational event.

These clones were subsequently screened for inactivation of *pfl* by measuring hydrogen production.

Chromosomal integration of *Z. mobilis* genes by using a circularized DNA fragment. The vector, pLOI510, was constructed to allow direct selection for the integration of *Z. mobilis pdc* and *adhB* genes into the *pfl* region of the chromosome by using a DNA fragment which lacks a replicon (Fig. 1B). The *cat* gene including its own promoter was purified as a 1.4-kbp *Hha*I fragment from pBR325 (24). After treatment with Klenow polymerase, this fragment was ligated into the Klenow-treated *Bam*HI site downstream from the *adhB* gene in pLOI295. The resulting construct, pLOI515, was selected such that *cat* transcription was oriented in the same direction as *pdc* and *adhB*. This plasmid was digested with *Sal*I and partially digested with *Eco*RI. After treatment with Klenow polymerase to generate blunt ends, a blunt *Bam*HI linker (dCCGGATCCGG) was ligated to both ends and the 4.6-kbp fragment was purified on an agarose gel. This fragment containing *cat* and promoterless *pdc* and *adhB* genes was digested with *Bam*HI and ligated into the *Bam*HI polylinker site of a pUC19 derivative

(pLOI505) in which the *Sal*I site had been deleted to produce pLOI506. Both the *Eco*RI and *Sal*I sites were regenerated on the ends of this fragment in pLOI506. The *Sal*I site upstream from *cat* was removed by digestion with *Sal*I, Klenow treatment, and ligation to produce pLOI508. The 4.6-kbp *Bam*HI fragment of pLOI508 containing *cat* and the *Z. mobilis* genes was inserted into the *Bam*HI site of *pfl'* on pLOI513, preserving the same direction of transcription to produce pLOI510.

*Sal*I digestion of pLOI510 produced an 8.6-kbp fragment containing the promoterless *Z. mobilis* genes and an adjacent *cat* gene (with a native promoter) within a promoterless *pfl'* and a 4-kbp fragment containing the replication genes. Approximately 3 μg of this 8.6-kbp fragment was purified by gel electrophoresis and ligated to itself to form closed circles. This circularized DNA was used to transform ATCC 11303 with selection for a single homologous recombination event which confers resistance to 20 μg of Cm per ml. Transformants were screened for expression of *adhB* on aldehyde indicator plates. Small-scale DNA preparations were tested for their ability to transform *E. coli* TC4 to confirm the absence of plasmid.

Insertion of *recA* and *frd* mutations. The *recA* mutation in *E. coli* JC10240 was transferred to strains KO4 and KO5 by conjugation with selection for resistance to both Cm and tetracycline (nearby *srl*::Tn*10*). Co-inheritance of the *recA* phenotype was confirmed by increased UV sensitivity.

An *E. coli* Hfr strain capable of mobilizing the *frd* mutation was constructed by transducing the *frd* mutation from DW12 [*zid*::Tn*10* Δ(*frdABCD*)] into strain KL282, with selection for Tn*10* (3). *frd* mutants were identified among the tetracycline-resistant transductants by loss of fumarate reductase activity (27). The resulting Hfr strain, SE1706, was used to conjugate the *frd* deletion into strain KO4.

Tn*10* was deleted from these mutants by selection on a modified fusaric acid medium (4, 21). This medium contained (per liter) 15 g of agar, 5 g of tryptone, 5 g of yeast extract, 20 g of glucose, 10 g of NaCl, 50 mg of chlorotetracycline, 10 g of NaH_2PO_4, 12 mg of fusaric acid plus $ZnCl_2$ (10 mM). Stocks of chlorotetracycline (12.5 mg/ml) and fusaric acid (2 mg/ml) were prepared in 70% ethanol. Complex medium components containing chlorotetracycline, sodium phosphate, and zinc chloride were autoclaved separately and mixed after cooling. Antibiotics in 70% ethanol are self-sterilizing. For selection of Tn*10* loss, serial dilutions of log-phase cultures were spread on fusaric acid plates and incubated overnight at 37°C. Resulting colonies were streaked for isolation on additional fusaric acid plates and tested for the loss of tetracycline resistance. Three strains were constructed, KO10 (*recA*), KO11 (*frd*), and KO12 (*recA frd*).

Fermentation experiments. Fermentations were carried out in Luria broth supplemented with 10% (wt/vol) glucose or 8% (wt/vol) xylose. Fleakers (500 ml; Fisher Scientific Co., Orlando, Fla.) containing 350 ml of the medium were equipped with a pH electrode, gas outlet, and sampling port through appropriately drilled rubber caps. A Jenco model 3671 pH controller (Whatman Lab Sales, Hillsboro, Ore.) was used to maintain a pH of 6.0 by the addition of base (2 N KOH). Batch fermentations were carried out in duplicate at 30°C and were stirred continuously by a 1.25-in. (3.97-cm) star-shaped magnetic bar (100 rpm).

Inocula were grown overnight at 30°C from isolated colonies in unshaken flasks. Fermentations were inoculated to an initial optical density at 550 nm of approximately 1.0 (330 mg [dry weight] of cells per liter).

Cell growth was monitored turbidimetrically at 550 nm by using a Bausch & Lomb Spectronic 70 spectrophotometer. Ethanol was measured by gas-liquid chromatography as previously described (10). Values for conversion efficiency were corrected for fermentation volume changes caused by the addition of base, and it was assumed that all sugar initially added had been metabolized. Volumetric productivities and specific productivities were estimated during the early stages of fermentation (6 to 24 h) and represent maximum values. All fermentation data in tables and in figures represent averages from two or more batch fermentations.

Analysis of volatile and nonvolatile acids in culture. Samples were removed for organic acid analyses after 72 h of fermentation. Volatile and nonvolatile acids were measured by gas-liquid chromatography with a Series 580 gas chromatograph (Gow-Mac Instrument Co., Bridgewater, N.J.) connected to a Hewlett-Packard 3390A integrator (12). Nonvolatile acids were converted to methyl esters prior to analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cells were grown for 24 h under the conditions of the fermentation experiments, chilled to 0°C, harvested by centrifugation (7,000 × *g*, 10 min), washed twice with 1/3 volume of 5 mM sodium phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol, and stored frozen at −20°C. Cell pellets were resuspended in an equal volume of buffer and broken by two passages through a French pressure cell at 20,000 lb/in^2 (20). Membranes were removed by centrifugation for 90 min at 100,000 × *g*. The supernatants were separated with a Mini-Protein II electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif.) by using an 8% acrylamide denaturing SDS gel (17). Protein was measured with the Bradford reagent (5). Approximately 20 μg of protein was loaded into each lane.

Hydrogen production. PFL cleaves pyruvate to produce acetyl CoA and formate. In *E. coli*, formate is rapidly degraded to produce a mixture of carbon dioxide and hydrogen. Thus, the production of hydrogen during anaerobic growth can be used to infer PFL function. One-milliliter cultures (Luria broth containing 5% glucose) were grown for 15 h at 37°C in stoppered culture tubes (12 by 75 mm) under a nitrogen atmosphere. Hydrogen produced during incubation was measured by gas chromatography with a thermal conductivity detector as previously described (27).

Enzyme activity. PDC activity was measured in heat-treated French press extracts (described above) as described previously (8). Heat treatment was used to inactivate competing native enzymes which complicate measurements of PDC activity in recombinant *E. coli*.

RESULTS

Chromosomal integration of *Z. mobilis pdc* and *adhB* genes. Two approaches were used to construct strains of *E. coli* ATCC 11303 in which the *Z. mobilis* genes for ethanol production were integrated into the *pfl* gene. The first involved a derivative of the temperature-conditional integration vector developed by Hamilton et al. (11). After transformation of ATCC 11303 with pLOI543 followed by temperature-based selection and enrichment for two homologous recombination events, 64.5% of the colonies were Cm sensitive, indicating loss of the plasmid. Of these Cm-sensitive clones, 5.9% formed pink colonies on aldehyde indicator plates, showing the presence of *Z. mobilis* ADHII. Two clones (KO1 and KO2) were selected for further study.

By using the second approach, a Cm-resistant transformant designated strain KO3 was obtained as a result of a single homologous recombination event by the integration of a circularized *Sal*I fragment from pLOI510. This clone also formed pink colonies identical to KO1 and KO2 on aldehyde indicator plates.

On aldehyde indicator plates, the control, ATCC 11303, formed white colonies (negative). ATCC 11303(pLOI297) formed intensely red colonies, indicating high-level expression of *Z. mobilis adhB*, and is an excellent strain for ethanol production (1).

Subsequently, the isolation of a vector from KO3 was attempted by alkaline SDS lysis (26). No vector was visible in agarose gels after staining with ethidium bromide. This preparation was also tested in transformation experiments with TC4 as the host with selection for Cm resistance. No transformants were recovered, confirming the absence of vector.

Hydrogen production as a measure of PFL activity. The parent organism, ATCC 11303, produced 19 μmol of hydrogen per tube during growth with L broth containing 5% glucose. An excellent ethanologenic recombinant (1), ATCC 11303(pLOI297) produced 1.9 μmol of hydrogen per tube. The reduction of hydrogen production in this recombinant is attributed to competition by the high levels of *Z. mobilis* PDC, which divert pyruvate to acetaldehyde instead of acetyl CoA and formate, the immediate precursor for hydrogen. No hydrogen was produced by strains KO1 or KO2, indicating that the native *pfl* gene had been inactivated by homologous recombination with DNA from plasmid pLOI543. Strain KO3 produced hydrogen (16 μmol per tube) at a level only slightly below that of the parent, consistent with the presence of a functional *pfl* gene. Insertion of the circular 8.6-kbp fragment from pLOI510 (Fig. 1B) into the *pfl* gene obviously resulted in gene duplication from homologous recombination, retaining pfl^+ with the *Z. mobilis* genes. The low level of *Z. mobilis* gene expression (pink colonies on aldehyde test plates) and Cm resistance confirms this phenotype.

Fermentation by strains KO1, KO2, and KO3. Figure 2A and B shows a comparison of fermentations by strains KO2 and ATCC 11303(pLOI297) with 10% glucose. Strain KO1 was identical to KO2 and is not shown. These new strains were very poor producers of ethanol, as indicated by the low volumetric productivity and ethanol yield (Table 2). Cell yield was limited to less than 30% of ATCC 11303(pLOI297), and only 4 g/liter of ethanol was produced after 72 h. Although strain KO3 was slightly better than KO1 and KO2, it still produced less than 25% of that of the control strain, ATCC 11303(pLOI297) (Fig. 2C and D; Table 2). Large volumes of base were consumed for the maintenance of pH during fermentations by these three new strains, indicating excessive production of acidic fermentation products.

Amplification of integrated *pdc* and *adhB* genes. The pink phenotype observed on aldehyde indicator plates (strains KO1, KO2, and KO3) and the production of near-wild-type levels of hydrogen during fermentation by KO3 appeared to indicate insufficient expression of *Z. mobilis* genes for ethanol production. Two approaches were used to obtain mutants with higher levels of expression of the *Z. mobilis* genes. Direct mutation and selection on aldehyde indicator plates led to the isolation of a KO2 mutant designated KO20 which produced high levels of both *Z. mobilis* genes.

In the second approach, selection for resistance to high levels of Cm was used to enrich for spontaneous mutants of KO3 in the hope that some of these would also express higher levels of the *Z. mobilis* genes. Serial dilutions of an overnight culture were plated on Luria agar plates containing 2% glucose and 600 μg of Cm per ml. Large, raised colonies which are indicative of high-level expression of *Z. mobilis pdc* and *adhB* (13) were observed after overnight incubation at a frequency of about 10^{-5}. All of these colonies exhibited a dark-red phenotype on aldehyde indicator plates identical to that of ATCC 11303(pLOI297), an excellent ethanol producing strain (1). Two mutants were selected for further study, strains KO4 and KO5. The lack of vector in these strains was again confirmed by the failure of DNA preparations from KO4 and KO5 to transform TC4 during selection for Cm resistance and by the absence of vector in agarose gels stained with ethidium bromide.

Fermentation by strains KO4, KO5, and KO20. Figure 2C and D illustrates the fermentation of 10% glucose by KO4, KO5, and KO20. Results with KO4, KO5, and KO20 were essentially identical, and only KO4 has been plotted. Growth, cell yield, and ethanol yield by these improved constructs were almost equivalent to those of the plasmid-based construct, ATCC 11303(pLOI297) (Table 2). Although the rate of ethanol production as indicated by volumetric productivities from the early stages and by the level of ethanol achieved after 30 h was lower than that of ATCC 11303(pLOI297), theoretical yields with KO4, KO5, and KO20 were higher and exceeded 100% on the basis of added glucose. This higher yield during slower fermentation reflects the cocatabolism of complex nutrients to pyruvate and thus to ethanol. These complex nutrients serve as the primary nitrogen source for biosynthesis. Continued deamination caused an increase in the pH with KO4, KO5, KO20, and ATCC 11303(pLOI297) after the sugars were exhausted. The resulting rise in pH provided a convenient method to monitor sugar exhaustion.

The fermentation of 8% xylose by strain KO4 and ATCC 11303(pLOI297) were also compared (Fig. 2E and F). KO4 was equivalent to the plasmid-based strain in ethanol yield, although the rate of ethanol production was slightly lower, as evidenced by the ethanol level after 30 h (Table 2).

Expression of *Z. mobilis* genes in recombinant *E. coli*. The specific activity of *Z. mobilis* PDC was measured in extracts of selected recombinant strains. PDC is relatively thermostable, and this enzymatic activity was measured in extracts after heat inactivation of native *E. coli* enzymes which complicate such measurements. No activity was detected in the control strain ATCC 11303. Both KO2 and KO3 produced low levels of activity (0.2 IU/mg of cell protein). Ten-fold-higher activity (2.1 IU/mg of cell protein) was present in extracts of the high Cm-resistant mutant, strain KO4. The specific activity of PDC in KO4 was comparable to that produced in the plasmid-based construct (2.7 IU/mg of cell protein) ATCC 11303(pLOI297) and similar to that in native *Z. mobilis* (15).

Protein extracts from these strains were also examined by SDS-PAGE (data not shown). Numerous changes in proteins were observed between ATCC 11303 and recombinant derivatives in addition to those attributable to *Z. mobilis* genes. *Z. mobilis* PDC exhibits an apparent molecular weight of 60,000 on SDS-PAGE (8). Consistent with measurements of enzymatic activity, KO4 and ATCC 11303(pLOI297) contained higher levels of a protein band in the 60,000-molecular-weight region than were present in KO2 and KO3. This band was absent in the ATCC 11303 control. Many native proteins were found in the 38,000-molecular-weight region in which ADHII is located, obscuring differences in expression.

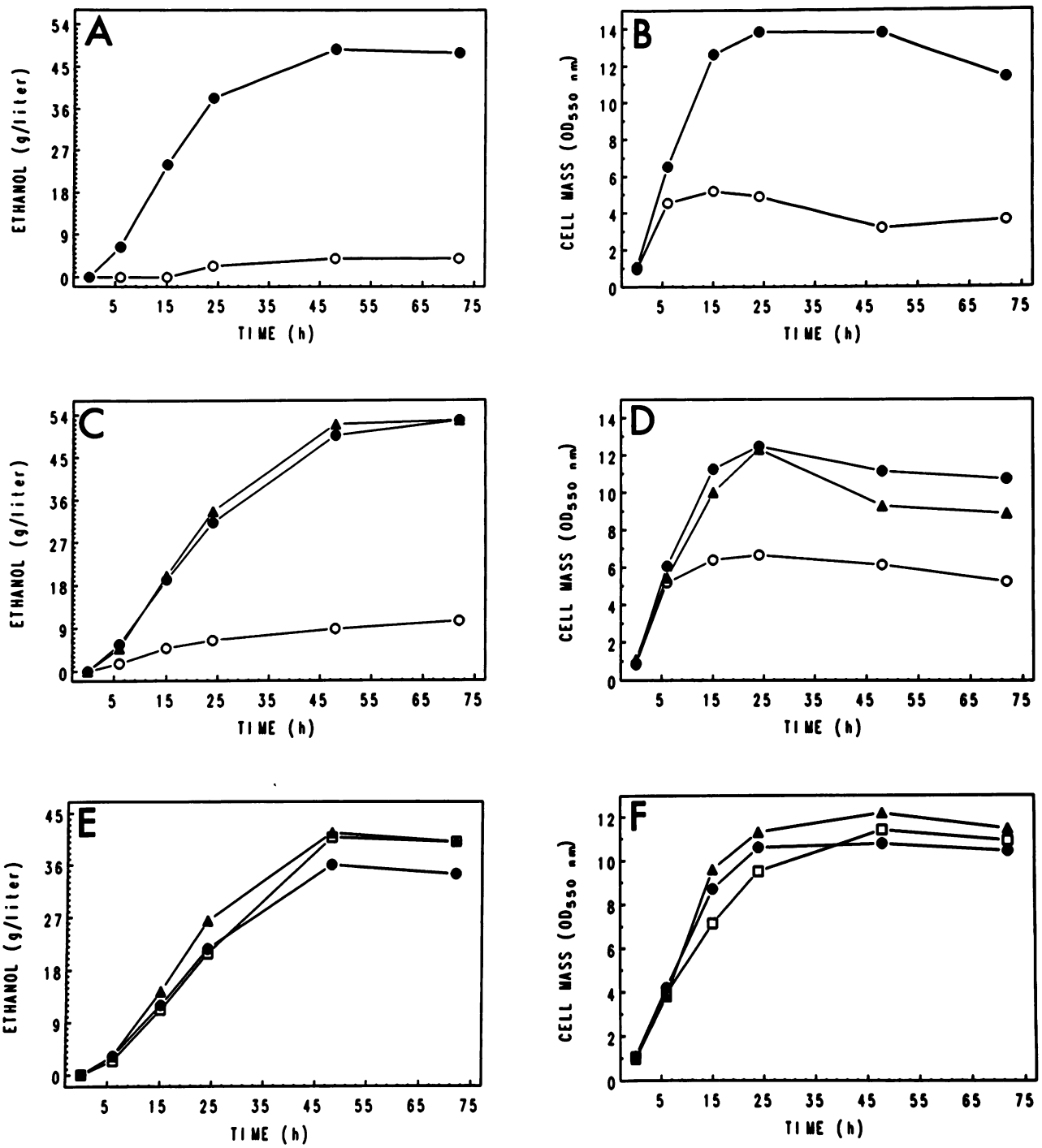

FIG. 2. Ethanol production (A, C, and E) and growth (B, D, and F) during batch fermentation. (A and B) Fermentation of 10% glucose. Symbols: ●, ATCC 11303(pLOI297); ○, KO2. (C and D) Fermentation of 10% glucose. Symbols: ○, KO3; ●, KO4; ▲, KO11. (E and F) Fermentation of 8% xylose. Symbols: ●, KO4; ▲, KO11; □, KO12.

Comparison of stability of *cat* and *Z. mobilis* genes in recombinant *E. coli*. ATCC 11303(pLOI297), KO4, and KO5 were serially transferred in Luria broth containing 10% glucose without antibiotics for more than 60 generations at 30°C. Appropriate dilutions of cultures were plated on Luria agar containing 2% glucose (without antibiotics) after 48 and 120 h. Colonies were screened on 2% glucose–Luria agar plates containing 40 μg of Cm per ml, 600 μg of Cm per ml, or 10 μg of tetracycline per ml and on aldehyde indicator plates. ADHII activity and high Cm resistance were fully maintained in KO4 and KO5 for 68 generations (Table 3), while 3% of ATCC 11303(pLOI297) cells had lost plasmid pLOI297, as evidenced by the loss of both tetracycline resistance and ADHII activity. Ethanologenic recombinants

TABLE 2. Ethanol production from glucose and xylose by recombinant strains of ATCC 11303[a]

Strain	Base (mmol/g of sugar)[b]	Ethanol yield		% Theoretical yield	VP (g/liter per h)[c]	30-h ethanol (g/liter)	Cell yield (g/g of sugar)
		g/liter	g/g of sugar				
10% Glucose							
pLOI297[d]	1.1	48.8	0.52	101	1.9	41.8	0.048
KO1	5.7	4.0	0.05	10	0.3	3.2	0.021
KO2	6.3	4.0	0.05	10	0.3	3.2	0.021
KO20	0.8	49.6	0.52	102	1.5	34.7	0.041
KO3	5.5	10.4	0.13	26	0.4	6.4	0.028
KO4	1.3	52.8	0.56	110	1.5	36.0	0.044
KO5	1.4	52.8	0.56	110	1.5	36.0	0.040
KO10 (*recA*)	1.1	51.2	0.54	107	1.8	38.0	0.041
KO11 (*frd*)	0.6	52.8	0.54	107	1.7	38.0	0.042
KO11 (*frd*)[e]	0	38.8	0.39	76	1.4	29	0.041
KO12 (*frd recA*)	1.1	54.4	0.57	112	1.2	30.4	0.035
8% Xylose							
pLOI297	1.4	36.0	0.47	94	1.0	30.0	0.050
KO4	1.6	36.0	0.47	94	1.1	25.6	0.047
KO11 (*frd*)	0.5	41.6	0.53	104	1.3	30.4	0.051
KO12 (*frd recA*)	0.64	40.8	0.53	103	1.1	26.0	0.048

[a] Calculations are based on total sugar added initially.
[b] Amount of base consumed to maintain a pH of 6.0 during fermentation.
[c] VP, Volumetric productivity.
[d] ATCC 11303(pLOI297).
[e] Fermentation conducted without pH control.

grew as thick, raised colonies on plates because of lack of auto-inhibition by acidic fermentation products (14). Loss of this trait was also readily apparent by the smaller colony morphology typical of wild-type *E. coli*.

Organic acid production by *E. coli* ATCC 11303 constructs. Large amounts of base were consumed by ethanologenic *E. coli* strains during fermentations (Table 2), indicating the production of acids as coproducts. Insertional inactivation of *pfl* by the *Z. mobilis* genes in KO1 and KO2 resulted in a 70% decrease in acetate production (Table 4). Since no hydrogen is produced by these two strains, this acetate is presumed to result from the catabolism of complex nutrients. Integration of the circular fragment containing the ethanologenic genes by a single recombinational event in strain KO3 did not inactivate *pfl*. Acetate levels in KO3 remained almost equivalent to the parental strain.

The high-level expression of the *pdc* and *adhB* genes in KO20 decreased the production of all three acidic fermentation products. A similar high level of expression in KO4 decreased the production of acetate and lactate to levels similar to those of the plasmid-based construct ATCC 11303(pLOI297), although large amounts of succinate remained. Succinate production was reduced by 95% by the introduction of a *frd* mutation into KO4 to produce KO11 (data not shown; Table 2). However, this low level of acid production was still sufficient to lower the pH of fermentations and reduce ethanol productivity when the pH control was omitted.

The elimination of fumarate reductase improved the rate of ethanol production, cell yield, and ethanol yield from xylose (Table 2). With glucose, volumetric productivity was increased by this mutation although ethanol yield remained essentially the same.

Introduction of a *recA* mutation. To minimize recombinational instability to develop alternative hosts for recombinant plasmids, a *recA* mutation was introduced into KO4 and KO11 to produce KO10 and KO12, respectively. The *recA* mutation did not affect acid production and fermentation in KO10, but it reduced growth and acetate production and slowed fermentation in KO12 (Fig. 2E and F; Tables 2 and 4). Although the basis of these effects in KO12 is unknown, they may represent secondary mutations created during construction.

TABLE 3. Stability of *cat* and ethanologenic genes in recombinant *E. coli*[a]

Recombinant	Percentage retaining traits (no. of generations)	
	Sample 1	Sample 2
ATCC 11303(pLOI297)	100 (37.3)	97 (67.3)[b]
KO4	100 (38.5)	100 (68.5)
KO5	100 (38.7)	100 (68.7)

[a] Cells were grown at 30°C in Luria broth containing 10% (wt/vol) glucose without antibiotic selection.
[b] Cm resistance and ethanol production genes were concomitantly lost.

TABLE 4. Production of acidic fermentation products

Recombinant	Organic acid (mM)[a]		
	Acetic acid	Lactic acid	Succinic acid
ATCC 11303	66	641	58
ATCC 11303(pLOI297)	21	32	49
KO1	18	673	62
KO2	18	602	57
KO3	69	525	66
KO4	22	29	70
KO10	18	20	73
KO11	4	32	2
KO12	6	40	2
KO20	4	60	2

[a] Values are the averages of two fermentations with 10% glucose fermentation sampled after 72 h.

DISCUSSION

In previously described ethanologenic strains of *E. coli*, *Z. mobilis pdc* and *adhB* genes were carried on multicopy plasmids, typically 15 to 700 copies per cell in pBR322- and pUC18-based constructs (26). This abundance of gene copies is responsible in large measure for the production of the high levels of *Z. mobilis* enzymes required to divert pyruvate to ethanol as the primary product of fermentation. To eliminate this need for a multicopy plasmid, the *pfl* gene was selected as a target for integration to provide high-level expression. Although *pfl* promoters are thought to be among the most active in *E. coli* (29), initial constructs (strains KO1, KO2, and KO3) in which the *Z. mobilis* genes were integrated provided insufficient levels of *Z. mobilis* enzymes and were poor producers of ethanol.

One of these constructs, KO3, included a *cat* gene from pBR325 with a separate promoter, downstream from the *pdc* and *adhB* genes. As a result of selecting spontaneous derivatives of this strain which were resistant to high levels of Cm, we obtained mutants in which expression of the *Z. mobilis* enzymes for ethanol production were simultaneously increased, as evidenced by the increase in PDC activity, stronger reaction on aldehyde indicator plates (ADHII), decreased acetate, and more efficient ethanol production. An analogous mutation in KO2 was obtained by direct selection for increased acetaldehyde production without antibiotic markers (KO20). These mutants, KO4, KO5, and KO20, were functionally equivalent to plasmid-based ATCC 11303(pLOI297) in terms of ethanol production from xylose and glucose.

The simultaneous improvement in ethanol production and resistance to high levels of Cm was surprising, since the *cat* gene included its own promoter and was located downstream from the proposed *adhB* transcriptional terminator (1, 9). Although increased expression could be a consequence of either gene duplication or a single mutational event, the single step nature, the successful isolation of analogous mutants from KO2 without antibiotic selection, and the stability of the new strains are most consistent with a mutational event in both KO3 and KO4. As a result of the integration of *pdc* and *adhB* genes into the chromosome, the stability of the new genetic trait for ethanol production was improved with 100% retention after 68 generations.

Significant amounts of succinate were produced as a coproduct by these integration constructs, despite their high yields of ethanol. Approximately 95% of this succinate was eliminated by deleting the *frd* gene. However, pH control was still required for maximal growth and ethanol production.

Elimination of the requirement for a plasmid to carry the *Z. mobilis* genes now provides additional opportunities for the commercial application of ethanologenic *E. coli*. Additional genes can be inserted into plasmids to direct the synthesis of recombinant proteins as valuable coproducts with no expected interference in ethanol productivity. A *recA* mutation was added to two of these strains to make them suitable hosts for the production of recombinant proteins.

ACKNOWLEDGMENTS

We thank S. R. Kushner for providing plasmid pMAK705 and G. Sawers and A. Bock for providing plasmid pHB4. We also thank Barbara Bachmann (Coli Genetic Stock Center) for providing strain KL282 and R. P. Gunsalus for sharing strain DW12.

These studies were supported in part by the Florida Agricultural Experiment Station; by grant FG05-86ER3574 from the Office of Basic Energy Science, U.S. Department of Energy; by grant 88-37233-3987 from the Alcohol Fuels Program, U.S. Department of Agriculture; and by grant GM37403 from the National Institutes of Health.

REFERENCES

1. **Alterthum, F., and L. O. Ingram.** 1989. Efficient ethanol production from glucose, lactose, and xylose by recombinant *Escherichia coli*. Appl. Environ. Microbiol. **55**:1943–1948.
2. **Beck, M. J.** 1989. Factors affecting efficiency of biomass fermentation to ethanol. Biotechnol. Bioeng. Symp. **17**:617–627.
3. **Blaut, M., K. Whittaker, A. Valdovinos, B. A. C. Ackrell, R. P. Gunsalus, and G. Cecchini.** 1989. Fumarate reductase mutants of *Escherichia coli* that lack covalently bound flavin. J. Biol. Chem. **264**:13599–13604.
4. **Bochner, B. R., H. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143**:926–933.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Christiansen, L., and S. Pedersen.** 1981. Cloning, restriction endonuclease mapping and post-translational regulation of *rspA*, the structural gene for ribosomal protein S1. Mol. Gen. Genet. **181**:548–551.
7. **Clark, D. P.** 1989. The fermentation pathways of *Escherichia coli*. FEMS Microbiol. Rev. **63**:223–234.
8. **Conway, T., Y. A. Osman, J. I. Konnan, E. M. Hoffmann, and L. O. Ingram.** 1987. Promoter and nucleotide sequences of the *Zymomonas mobilis* pyruvate decarboxylase. J. Bacteriol. **169**: 949–954.
9. **Conway, T., G. W. Sewell, Y. A. Osman, and L. O. Ingram.** 1987. Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. J. Bacteriol. **169**:2591–2597.
10. **Dombek, K. M., and L. O. Ingram.** 1986. Determination of the intracellular concentration of ethanol in *Saccharomyces cerevisiae* during fermentation. Appl. Environ. Microbiol. **51**:197–200.
11. **Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner.** 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol. **171**:4617–4622.
12. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
13. **Ingram, L. O., and T. Conway.** 1988. Expression of different levels of ethanologenic enzymes from *Zymomonas mobilis* in recombinant strains of *Escherichia coli*. Appl. Environ. Microbiol. **54**:397–404.
14. **Ingram, L. O., T. Conway, D. P. Clark, G. W. Sewell, and J. F. Preston.** 1987. Genetic engineering of ethanol production in *Escherichia coli*. Appl. Environ. Microbiol. **53**:2420–2425.
15. **Ingram, L. O., C. K. Eddy, K. F. Mackenzie, T. Conway, and F. Alterthum.** 1989. Genetics of *Zymomonas mobilis* and ethanol production. Dev. Ind. Microbiol. **30**:53–69.
16. **Jeffries, T. W., and H. K. Sreenath.** 1988. Fermentation of hemicellulose sugars and sugar mixtures by *Candida shehatae*. Biotechnol. Bioeng. **31**:502–506.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lin, E. C. C.** 1987. Dissimilatory pathways for sugars, polyols, and carbohydrates, p. 244–284. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*, vol. 1. American Society for Microbiology, Washington, D.C.
19. **Luria, S. E., and M. Delbruck.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
20. **Mackenzie, K. F., C. K. Eddy, and L. O. Ingram.** 1989. Modulation of alcohol dehydrogenase isozyme levels in *Zy-*

momonas mobilis by iron and zinc. J. Bacteriol. **171**:1063–1067.
21. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145**: 1110–1112.
22. **Neale, A. D., R. K. Scopes, and J. M. Kelly.** 1988. Alcohol production from glucose and xylose using *Escherichia coli* containing *Zymomonas mobilis* genes. Appl. Microbiol. Biotechnol. **29**:162–167.
23. **Ohta, K., F. Alterthum, and L. O. Ingram.** 1990. Effects of environmental conditions on xylose fermentation by recombinant *Escherichia coli*. Appl. Environ. Microbiol. **56**:463–465.
24. **Prentki, P., F. Karch, S. Iida, and J. Meyer.** 1981. The plasmid cloning vector pBR325 contains a 482 base-pair-long inverted duplication. Gene **14**:289–299.
25. **Rodel, W., W. Plaga, R. Frank, and J. Knappe.** 1988. Primary structures of *Escherichia coli* pyruvate formate-lyase and pyruvate-formate-lyase-activating enzyme deduced from the DNA nucleotide sequences. Eur. J. Biochem. **177**:153–158.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Sankar, P., and K. T. Shanmugam.** 1988. Biochemical and genetic analysis of hydrogen metabolism in *Escherichia coli*: the *hydB* gene. J. Bacteriol. **170**:5433–5439.
28. **Sawers, G., and A. Bock.** 1988. Anaerobic regulation of pyruvate formate-lyase from *Escherichia coli* K-12. J. Bacteriol. **170**:5330–5336.
29. **Sawers, G., and A. Bock.** 1989. Novel transcriptional control of the pyruvate formate-lyase gene: upstream regulatory sequences and multiple promoters regulate anaerobic expression. J. Bacteriol. **171**:2485–2498.
30. **Skoog, K., and B. Hahn-Hagerdal.** 1988. Xylose fermentation. Enzyme Microbiol. Technol. **10**:66–80.